AD-A060 219 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/1
CROSS-REACTIONS BETWEEN TRYPTIC POLYPEPTIDES OF STAPHYLOCOCCAL --ETC(U)
SEP 78 L SPERO, B A MORLOCK

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CROSS-REACTIONS BETWEEN TRYPTIC POLYPEPTIDES OF STAPHYLOCOCCAL ENTEROTOXINS B AND C

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Running title: Heterologous Enterotoxin Peptide Cross-Reactions

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SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE *(and Subtitle)*

(6) Cross-Reactions Between Tryptic Polypeptides of Staphylococcal Enterotoxins B and C

5. TYPE OF REPORT & PERIOD COVERED

(9) Interim rept.

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)

(10) Leonard Spero and Beverly A. Morlock

8. CONTRACT OR GRANT NUMBER(s)

9. PERFORMING ORGANIZATION NAME AND ADDRESS

SGRD-UIP-A
U.S. Army Medical Research Institute of Infectious Diseases, Ft Detrick
Frederick, MD 21701

405 059

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

61102
(16) 3M161102BS03-00-006
(14) 00

11. CONTROLLING OFFICE NAME AND ADDRESS

U.S. Army Medical REsearch and Development Command, Office of the Surgeon General
Department of the Army, Washington, DC 20314

12. REPORT DATE

(11) Sep 78

13. NUMBER OF PAGES

22 pages

14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*

15. SECURITY CLASS. *(of this report)*

UNCLASSIFIED

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited.

(12) 25 p.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

Reprints bearing assigned AD number will be forwarded upon receipt. To be submitted for publication in The Journal of Immunology.

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Enterotoxins, cross reaction, antigenic determinants, serologic cross reactivity.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

See other side.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

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SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

The strong cross-reactions demonstrated for staphylococcal enterotoxins B (SEB) and C_1 (SEC_1) by measurement of antigen-binding capacity (J. Immunol. 120:86, 1978) were reflected in well-defined polypeptides obtained by limited tryptic digestion from SEB and SEC_1 (J. Biol. Chem. 248:7289, 1973; 251:5580, 1976). Two antigenic determinants on each enterotoxin were capable of reacting with heterologous antibody, one on the first 57 amino acids and one on the last 150 residues of the polypeptide backbone. The larger, carboxyl terminal polypeptides bound efficiently to homologous antiserum but about two orders of magnitude less efficiently to heterologous antibody. The amino terminal peptides showed only weak homologous binding but nearly comparable heterologous binding. It is proposed that the determinant on the amino terminal polypeptides is largely responsible for the strong reciprocal binding of the intact enterotoxins and that their low antigen-binding capacity is due to a random or a structurally distorted conformation in solution.

HETEROLOGOUS ENTEROTOXIN PEPTIDE CROSS-REACTIONS

CROSS-REACTIONS BETWEEN TRYPTIC POLYPEPTIDES OF STAPHYLOCOCCAL ENTEROTOXINS B AND C[1]

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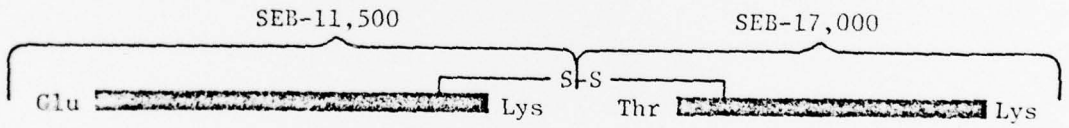
SUMMARY

The strong cross-reactions demonstrated for staphylococcal enterotoxins B (SEB) and C_1 (SEC_1) by measurement of antigen-binding capacity (J. Immunol. 120:86, 1978) were reflected in well-defined polypeptides obtained by limited tryptic digestion from SEB and SEC_1 (J. Biol. Chem. 248:7289, 1973; 251:5580, 1976). Two antigenic determinants on each enterotoxin were capable of reacting with heterologous antibody, one on the first 57 amino acids and one on the last 150 residues of the polypeptide backbone. The larger, carboxyl terminal polypeptides bound efficiently to homologous antiserum but about two orders of magnitude less efficiently to heterologous antibody. The amino terminal peptides showed only weak homologous binding but nearly comparable heterologous binding. It is proposed that the determinant on the amino terminal polypeptides is largely responsible for the strong reciprocal binding of the intact enterotoxins and that their low antigen-binding capacity is due to a random or a structurally distorted conformation in solution.

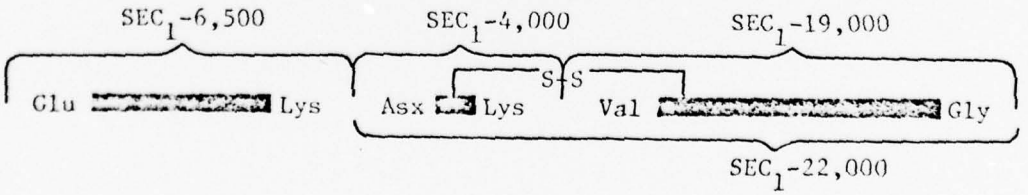
The staphylococcal enterotoxins have been classified on the basis of serologic individuality. However, cross-reaction between types A and E has been demonstrated (1) and clearly the lack of cross-reaction does not preclude the presence of common antigenic determinants among the various types. We have described a strong reciprocal reaction between SEB[2] and SEC_1 with each other's antibody by measurement of antigen-binding capacity (2).

SEB and SEC_1 undergo limited specific digestion by trypsin (3, 4). The primary cleavage in both instances occurs at a site on the polypeptide chain interior to the single disulfide loop of these proteins. A secondary cleavage also occurs, which for SEC_1 goes rapidly to completion. Singly cleaved enterotoxin B, SEB-T, and the doubly cleaved enterotoxin C_1, SEC_1-T_2, may be represented in linear form. The indicated molecular weights of the SEB-T polypeptides are known precisely (3) but since the amino acid sequence of SEC_1 has not been established, the molecular weights associated with the SEC_1-T_2 polypeptides are the values calculated from amino acid analysis (4):

SEB-T



SEC_1-T_2



SEB-11,500 and SEB-17,000 have been shown to contain antigenic sites for antibody to SEB (5). Similarly SEC_1-6,500 and SEC_1-22,000 (and its constituent fragment, SEC_1-19,000) possess determinants for antibody to SEC_1 (6). SEC_1-4,000 does not react with antibody to native SEC_1 (6). We have now investigated the cross-reaction of these peptides with antiserum to the heterologous enterotoxin; the presence of two common binding sites on structurally analogous polypeptides from widely separated regions of the molecules is reported here.

MATERIALS AND METHODS

Preparation of enterotoxins and tryptic peptides. The enterotoxins were produced and isolated by published methods as modified in our laboratory (3, 4). All the polypeptides were isolated by repeated gel chromatography on Sepharose 6B in 6 M guanidine hydrochloride, SEC_1-6,500 and SEC_1-22,000 directly from SEC_1-T_2, and SEB-11,500 and SEB-17,000 from SEB-T and SEC_1-4,000 and SEC_1-19,000 from SEC_1-22,000 after reduction and alkylation with iodoacetamide. By polyacrylamide gel electrophoresis in sodium dodecyl sulfate all the polypeptides were essentially homogeneous. Stable solutions of all the peptides were obtained by dilution of their concentrated solutions in 6 M guanidine hydrochloride into 10% bovine serum albumin.

Preparation of antisera. Antisera to SEB and SEC_1 were prepared by intracutaneous injection without adjuvant in New Zealand white rabbits.[3] A regimen based on that developed by Silverman was employed (7). Pools were made from those sera giving identical Ouchterlony titers. Rabbit antiserum for SEC_1-22,000 was produced by intramuscular inoculation of three 100-μg doses of polypeptide at weekly intervals

in 10% rabbit serum albumin. Most of the serum used in this study was from a bleeding of a single rabbit made one week after a second course of immunization administered 3 months after the first series of injections. The specific immunoglobulin content of these antisera was determined by the antigen-binding capacity assay assuming that in antigen excess all of the antigen was present as Ag_2Ab: anti-SEB, 0.66 mg/ml; anti-SEC_1, 0.46 mg/ml; anti-SEC_1-22,000, 0.073 mg/ml.

Labeling of enterotoxins and tryptic polypeptides. All labeling was carried out by the gaseous diffusion method of Gruber and Wright (8) with ^{125}I. Because some of the polypeptides had very poor solubility in aqueous buffers all of them were dissolved in 6 M guanidine hydrochloride during labeling. Unbound radioisotope was removed by dialysis against 6 M guanidine hydrochloride. The concentrations of the labeled enterotoxins and polypeptides were determined from their absorbance at 277 nm. Where extinction coefficients have not been determined experimentally they were calculated from the tyrosine and tryptophan content of the peptides.

Antigen-binding capacity assay. A modification of the Farr technique (9) based on the ability of protein A-containing strains of Staphylococcus aureus to react specifically and with high affinity with the Fc portions of IgG was employed (10). Labeled antigen (20 to 100 ng in 500 µl) was added to 500-µl volumes of twofold serially diluted antiserum in phosphate buffered saline containing 0.5% bovine serum albumin. Dilution of the labeled enterotoxins was made in the same diluent but the 6 M guanidine hydrochloride solutions of the labeled polypeptides were diluted with a 10% albumin solution in buffered saline. To separate bound from unbound antigen a 10% suspension

of the Cowan I strain of S. aureus was added. For serum dilutions of 1:40 and above, 100 µl of the 10% suspension were adequate to precipitate all the immunoglobulin but for dilutions of 1:10 and 1:20, 500 µl were required and for 1:5 dilutions of serum a full milliliter was needed. The reaction tubes were treated and the calculation of percentage of antigen bound to antibody was made as previously described (2). In the calculations a correction was made for nonspecific precipitation by the bacterial cell suspension.

Fifty percent endpoints were estimated from log-log plots of the volume of antibody against the percent of antigen bound. These graphs were linear from about 20 to 80% bound and had nearly identical slopes. This latter property facilitated the endpoint estimation when an extrapolation from the data was required.

Circular dichroic spectra. A Jasco J-40 instrument equipped with a data processor was used to obtain circular dichroic spectra. The polypeptides were prepared for analysis by dialysis of concentrated solutions in 6 M guanidine hydrochloride against repeated changes of large volumes of phosphate-buffered saline. Sixty-four repetitive scans were run using the data processor at a time constant of 4s and with a resolution of 0.2 nm/step.

RESULTS AND DISCUSSION

Binding curves for the tryptic polypeptides with antiserum to SEB are presented in Figure 1 and with antiserum to SEC_1 in Figure 2. It is quite evident by comparison with the binding curves of the intact enterotoxins that the binding capacity of the larger fragments, SEC_1-22,000, SEC_1-19,000 and SEB-17,000, for each homologous antiserum is

very strong. Nearly complete precipitation of label was readily achieved at high dilutions of the antisera. Homologous reactions of the smaller polypeptides and heterologous reactions of all the polypeptides are considerably weaker. Indeed, to demonstrate binding it was necessary in several instances to reduce the concentration of labeled antigen some four- to fivefold and to increase the volume of antiserum added up to 100 µl/ml. The data in the figures have been normalized to equal amounts of labeled antigen; this is based on the assumption that over the range of antigen employed the ratio of antibody to antigen at the endpoint is independent of antigen level.

In addition to the binding curves shown in the figures, binding capacity was measured for all the tryptic polypeptides and for the two intact enterotoxins to antisera to SEB to SEC_1 and to SEC_1-22,000. When the 50% endpoint values were converted to molar antibody to antigen ratios the values shown in Table I were obtained. In these calculations the simplifying assumption was made that all the antigen bound at the endpoint was in the form Ag_2Ab (11) so that for bivalent antibody the molar ratio of antigen to antibody was 4. Efficacy of binding is inversely proportional to the tabular values. The most striking observation is that there are significant heterologous interactions and that the reciprocal reaction between the whole enterotoxins with each other's antiserum is reflected in their constituent polypeptides. Consider first the large polypeptides from the carboxyl terminal end of the amino acid backbone and comprising more than half the enterotoxin molecules. The 17,000 M_r polypeptide from SEB binds to anti-SEC_1 and the 22,000 M_r polypeptide and the 19,000 M_r polypeptide from SEC_1 bind to anti-SEB. The molar ratios of antibody to antigen of the

latter two polypeptides at the 50% endpoint are in the same ratio to each other for all three antisera suggesting that the cross-reacting determinant of SEC_1-22,000 is contained totally within SEC_1-19,000. This is supported by the lack of binding of SEC_1-4,000 to either anti-SEB or to anti-SEC_1. (The binding of SEC_1-4,000 to anti-SEC_1-22,000 is attributed to a response to a non-native determinant in that antiserum [6]). The superior binding capacity of SEC_1-22,000 may be ascribed to a higher degree of refolding to a native-like conformation.

Each of these three carboxyl terminal polypeptides binds about two orders of magnitude more efficiently to its homologous antiserum than to the heterologous antiserum. Thus the relative values were: 214 for SEC_1-22,000 (94/0.44); 191 for SEC_1-19,000 (287/1.5); and 102 for SEB-17,000 (224/2.2). A comparison of the binding of the structurally analogous polypeptides from the two enterotoxins, SEC_1-19,000 and SEB-17,000, with the same antiserum yields similar estimates of binding efficacy. With anti-SEC_1, SEB-17,000/SEC_1-19,000 was 149 (224/1.5) and with anti-SEB, SEC_1-19,000/SEB-17,000 was 130 (287/2.2). These crude estimates show quite clearly that an antigenic determinant exists on SEC_1-19,000 and SEB-17,000 which, although capable of reacting with heterologous antiserum, is not functionally identical. It is probably also reasonable to infer that they differ structurally in the native enterotoxins.

The very excellent binding of SEB-17,000 to the antiserum to SEC_1-22,000 was unexpected. The molar ratio was even lower than with the homologous antiserum (1.6 with anti-SEC_1-22,000 and 2.2 with anti-SEB). This must reflect reaction with an antibody population arising from non-native determinants on SEC_1-22,000. It also suggests that

SEB-17,000 and SEC_1-22,000 possess regions of common structure in addition to that responsible for the common determinant described above.

The binding capacity of the amino terminal polypeptides, the 6,500 M_r polypeptide from SEC_1 and the 11,500 M_r polypeptide from SEB was very much weaker than the larger carboxyl terminal polypeptides even to the homologous antiserum. However, the relative binding efficacy with heterologous antiserum was much greater than that observed with the larger polypeptides. The value for SEC_1-6,500 was only 4.1 (329/81). Similarly a comparison of the binding of the two smaller polypeptides with anti-SEB, SEC_1-6,500/SEB-11,500 was 1.7 (329/189). This suggests a high degree of similarity in the structure of this determinant in SEB and SEC_1, which is presumably contained in the first 57 amino acid residues of the amino acid sequence.

There was one apparent anomaly in these observations, the failure to find binding of SEB-11,500 to anti-SEC_1. To test the possibility that this was due to inadequate or improper folding of labeled SEB-11,500 upon dilution into 10% bovine serum albumin, the technique used for all the peptides in this study, several other methods of renaturation were attempted. In all but one there was no significant change. However, when the labeled antigen in 6 M guanidine hydrochloride was dialyzed directly against phosphate buffered saline a remarkable result was obtained (Table II). The affinity to anti-SEB was increased 80-fold and, where no reaction had previously been found with the anti-SEC_1-22,000 or with anti-SEC_1, excellent binding was now seen. We attribute this enormous change largely to aggregation of the antigen. This effect was reported earlier (5) and is attested to here by an increase in nonspecific precipitation with the S. aureus cells to about 40% of

the total radioactivity. The binding to the antiserum to the 22,000 M_r poplypeptide may again be due to the presence of an unfolded determinant in SEB-11,500. A similar effect was noted with SEC_1-4,000 (6) and it should be added that the carboxyl terminal sequence of SEB-11,500 is probably homologous with SEC_1-4,000. The essential point is that this experiment demonstrates the heterologous binding of SEB-11,500 to enterotoxin C_1 antiserum.

Thus we have demonstrated the presence of two antigenic determinants on SEB and SEC_1 capable of reacting with heterologous antibody.[4] It is, however, difficult to reconcile the weak heterologous binding of the larger polypeptides and the weak homologous binding of the smaller amino terminal peptides with the extremely high binding capacity of the intact enterotoxins for heterologous antibody. Only four times the amount of antibody is required to give equivalent binding with the heterologous antigen as with the homologous antigen for both enterotoxins (2).

One may invoke the concept of a native format determinant proposed by Sachs et al. (12). All the components of the determinant may be present in a limited length of the polypeptide chain but may exist in solution in equilibrium among a variety of random conformations. One factor in the overall binding constant is a conformational equilibrium constant. The larger polypeptides would appear to have a high conformational constant. They bound very well to the homologous antibody; the strong binding of SEC_1 to antiserum raised against SEC_1-22,000 is also supportive of this contention, it being well established that antibodies elicited by immunization with denatured proteins either fail to react, or do not react extensively, with the native protein (13). Moreover, we have shown that the circular dichroic spectra in the far

ultraviolet of the 22,000 M_r polypeptide and the 19,000 M_r polypeptide from SEC_1 are similar to that of the native enterotoxin (6) indicating significant refolding to a native-like conformation. Conversely the CD spectrum of SEC_1-6,500 indicated it to be in a random chain conformation (6) and this fragment would therefore have a comparatively low conformational constant accounting for its weak antigen-binding capacity.

The CD spectra of the SEB tryptic polypeptides (Figure 3) are not, however, consistent with this simple explanation. Both SEB-11,500 and SEB-17,000 have a sufficiently stable folding to provide spectra indicative of significant secondary structure. Furthermore, these spectra differ from those of the parent enterotoxin. The weak binding of SEB-11,500 and the good binding of SEB-17,000 may not then be interpreted in terms of conformational constants but perhaps as a reflection of the correctness of folding, the determinant of the larger peptide being in a relatively native conformation and SEB-11,500 having its determinant in a distorted conformation.

It is suggested that the weak heterologous binding of the larger polypeptides is due to an actual compositional difference in the SEB and SEC_1 determinants. The weak homologous binding of the amino terminal polypeptides is considered to be due to a random conformation for SEC_1-6,500 and to an incorrect folding for SEB-11,500. Finally, since the ratio of binding capacity of SEC_1-6,500 with anti-SEB compared to anti-SEC_1 is only 1:4.1 and the ratio for SEB-11,500 with anti-SEC_1 compared to anti-SEB is only 1:7.1, it is proposed that the determinant on these fragments is primarily responsible for the excellent heterologous binding in the intact enterotoxins.

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TABLE 1

Binding of enterotoxin B and C_1 and tryptic peptides derived from them with antisera

Antigen	Molar Ratio of Total Antibody to Labeled Antigen at 50% Endpoint		
	Anti-SEC_1-22,000	Anti-SEC_1	Anti-SEB
SEC_1[a]	0.57	0.25[b]	1.0
SEC_1-6,500	NBE[c]	81[d]	329[d]
SEC_1-22,000	0.25[b]	0.44	94[d]
SEC_1-19,000	1.2	1.5	287[d]
SEC_1-4,000	4.3[d]	NBE	NBE
SEB	6.6	1.0	0.25[b]
SEB-11,500	NBE	NBE	189[d]
SEB-17,000	1.6	224[d]	2.2

[a]Data for SEC_1 peptides against anti-SEC_1-22,000 and anti-SEC_1 are from our report (6).

[b]Fixed by computational assumptions.

[c]No binding evident.

[d]Endpoint estimated by linear extrapolation of log-log plot.

TABLE II

Effect of method of renaturation upon binding of SEB-11,500 to antisera

Method of Renaturation	Molar Ratio of Total Antibody to Labeled Antigen at 50% Endpoint		
	Anti-SEC_1-22,000	Anti-SEC_1	Anti-SEB
Dilution in 10% bovine serum albumin	NBE[a]	NBE	189[b]
Dialysis against phosphate buffered saline	1.8	17	2.4

[a] *No binding evident.*

[b] Endpoint estimated by linear extrapolation of log-log plot.

FOOTNOTES

[1]The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

[2]Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; SEC_1, the variant of staphylococcal enterotoxin C with the more alkaline isoelectric point.

[3]In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

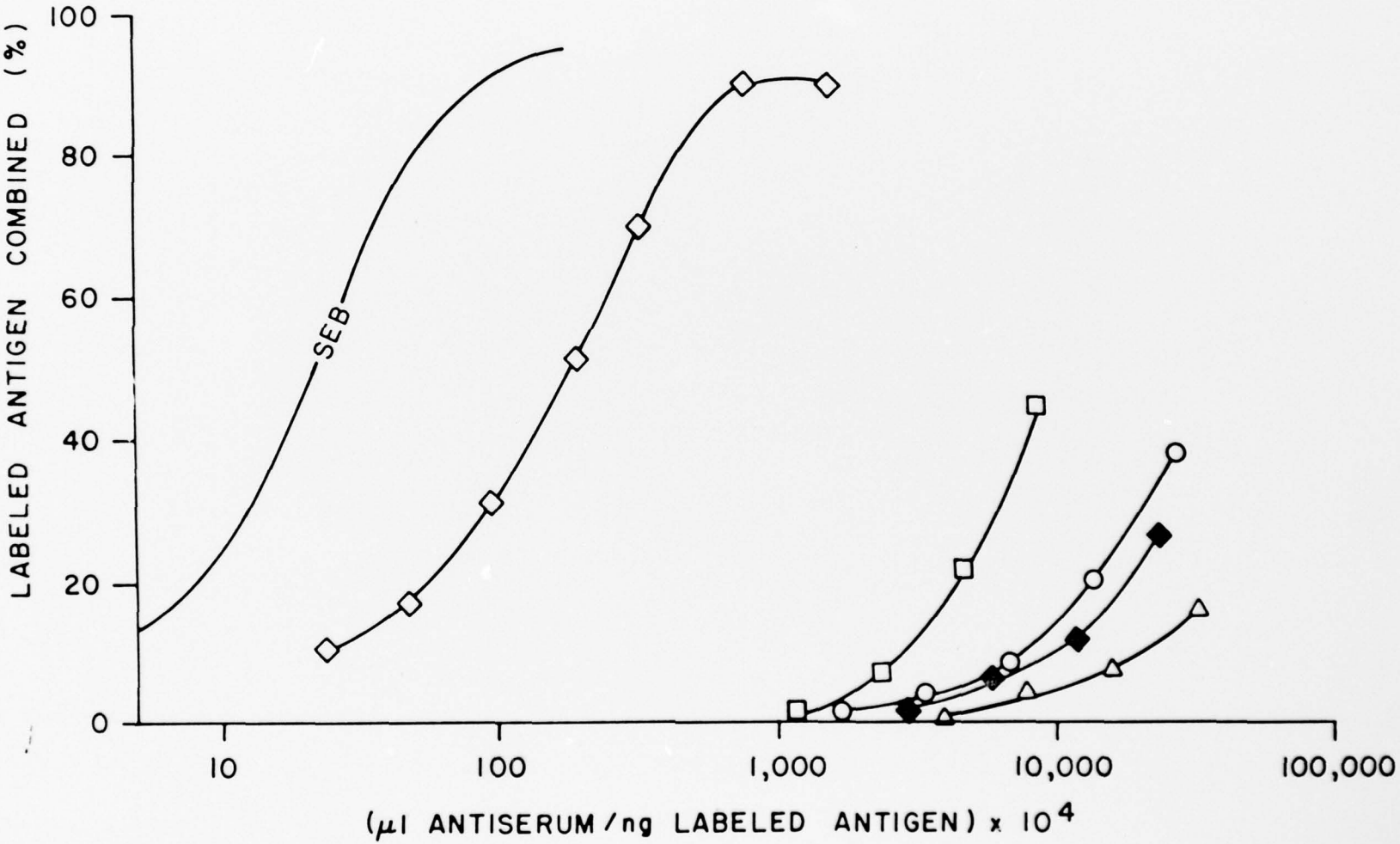
[4]The absence of cross-precipitation precludes the possibility of three cross-reacting determinants.

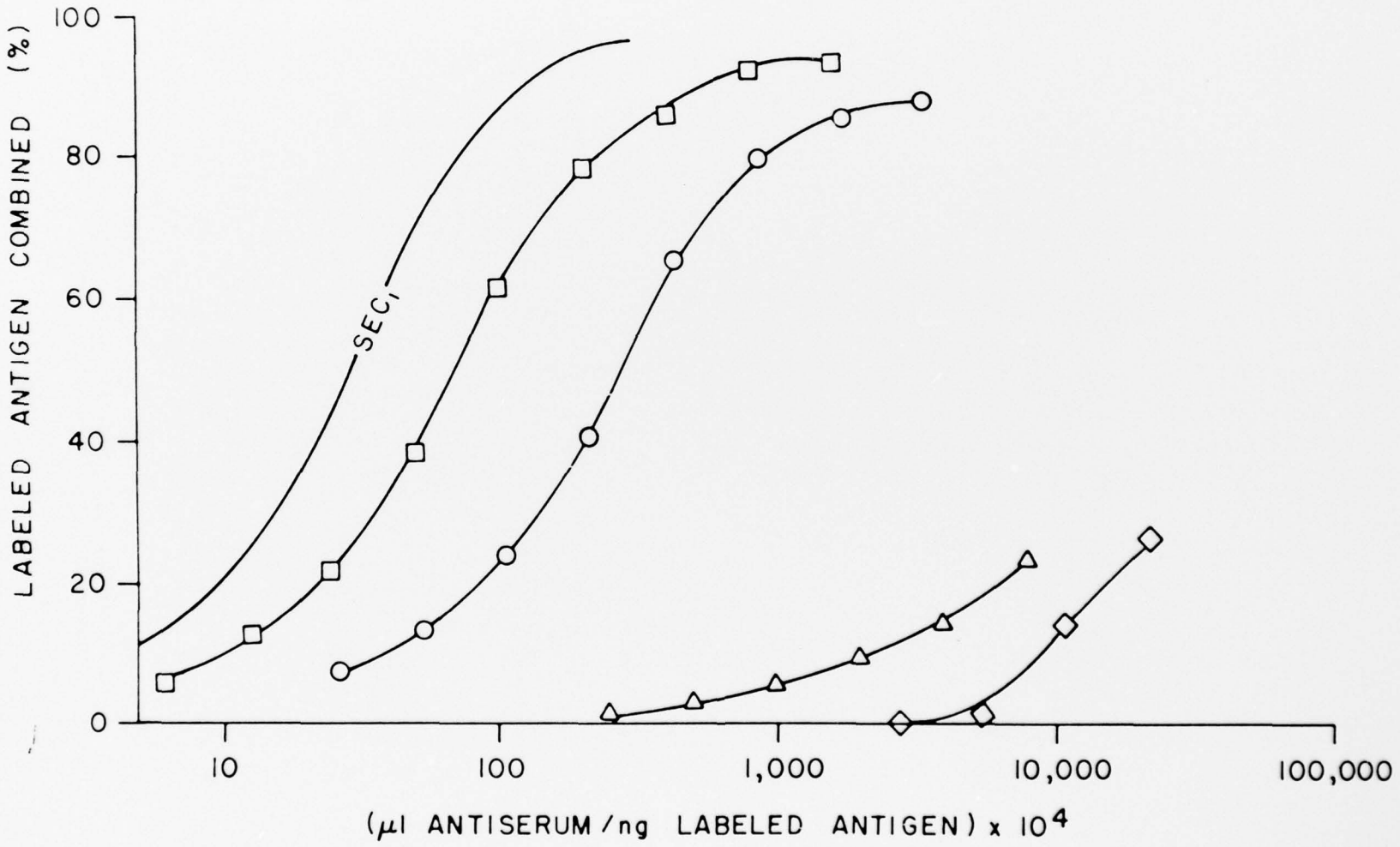
LEGENDS FOR FIGURES

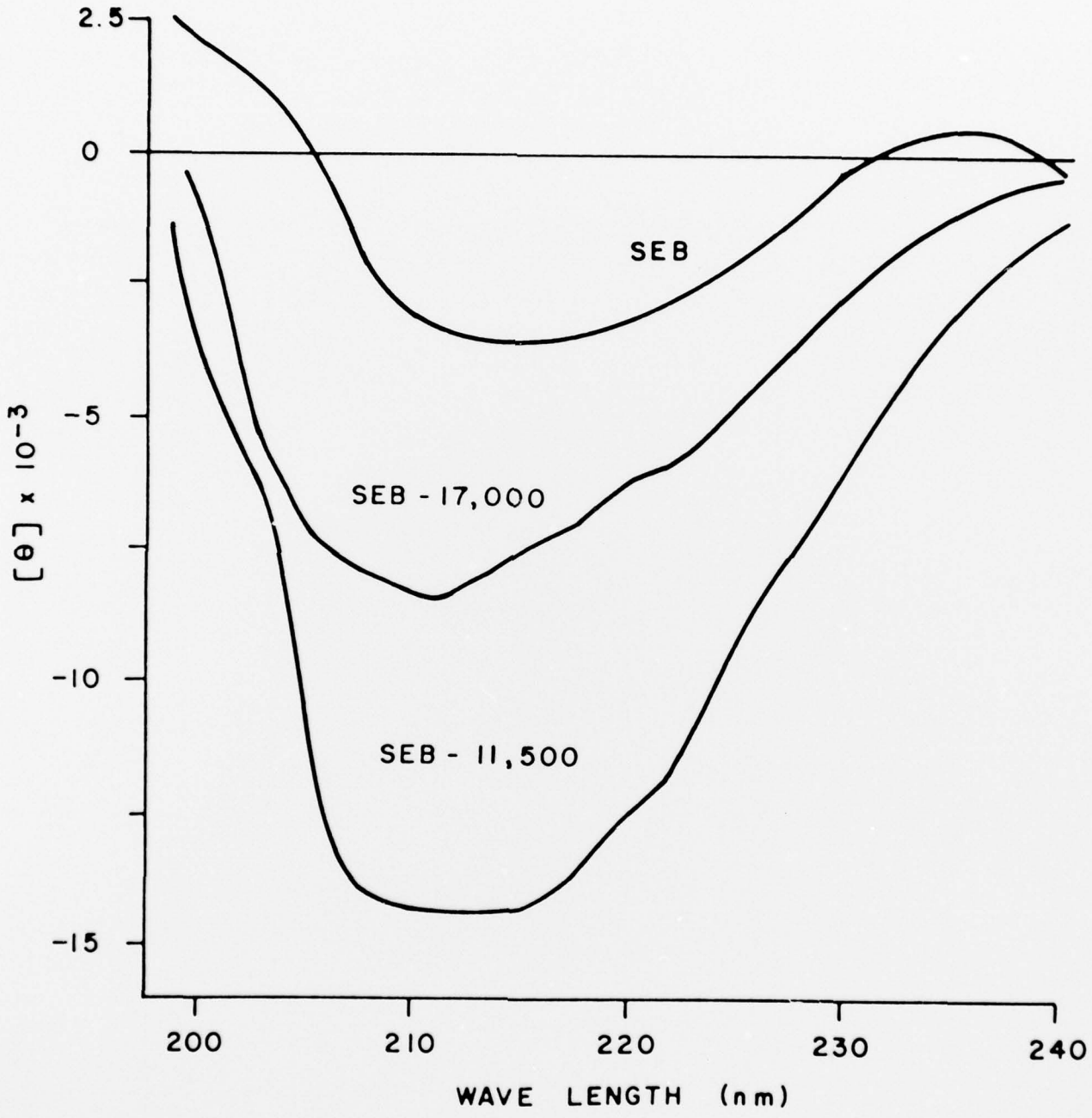
Figure 1. Binding of ^{125}I-labeled enterotoxin B and ^{125}I-labeled polypeptides derived from enterotoxin B and enterotoxin C_1 by tryptic digestion to rabbit antiserum to enterotoxin B. The data have been normalized to equal amounts of labeled antigen. The curve for enterotoxin B is a composite of a large number of experiments. (◇) SEB-17,000; (□) SEC_1-22,000; (○) SEC_1-19,000; (◈) SEB-11,500; (△) SEC_1-6,500.

Figure 2. Binding of ^{125}I-labeled enterotoxin C_1 and ^{125}I-labeled polypeptides derived from enterotoxin C_1 and enterotoxin B by tryptic digestion to rabbit antiserum to enterotoxin C_1. The data have been normalized to equal amounts of labeled antigen. The curve for enterotoxin C_1 is a composite of a large number of experiments. (□) SEC_1-22,000; (○) SEC_1-19,000; (△) SEC_1-6,500; (◇) SEB-17,000.

Figure 3. Circular dichroic spectra in the far-ultraviolet of enterotoxin B and the polypeptides derived from it by trypsin digestion. SEB was analyzed in a 0.1-mm cell at 1.09 mg/ml; SEB-11,500 and SEB-17,000 were run in a 0.5-mm cell at concentrations of 0.25 mg/ml.


LABELED ANTIGEN COMBINED (%)
100
80
60
40
20
0
SEB
10
100
1,000
10,000
100,000
(μl ANTISERUM / ng LABELED ANTIGEN) x 10^4


LABELED ANTIGEN COMBINED (%)
100
80
60
40
20
0
SEC1
10
100
1,000
10,000
100,000
(μl ANTISERUM/ng LABELED ANTIGEN) x 10^4


2.5
0
-5
-10
-15
[θ] x 10⁻³
SEB
SEB - 17,000
SEB - 11,500
200
210
220
230
240
WAVE LENGTH (nm)